

# PATENT SPECIFICATION

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NO DRAWINGS

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## (54) VITAMIN B<sub>12</sub> CONCENTRATES

(71) We, RICHTER GEDEON VEGYESZETI GYAR RT., a Body Corporate under the laws of Hungary, of 21 Gyomroi ut Budapest X., Hungary, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

10 The invention relates to the recovery of vitamin B<sub>12</sub> and other microbiologically active cobalamins from the liquid fermentation products of the cobalamin-producing fermentations, in the form of a  
 15 dry concentrate which can be utilized for the recovery of crystalline vitamin B<sub>12</sub> as well as for the preparation of feed-supplements containing vitamin B<sub>12</sub> and other biologically active cobalamins and  
 20 growth factors and also substantial amounts of proteins.

It is known that the vitamin B<sub>12</sub> and also the compositions containing vitamin B<sub>12</sub> are prepared usually in the following way:

25 In the fermentation products obtained e.g. by fermentations with methanobacteria or propionibacteria, the bacterium cells are destroyed to release into the liquid medium the vitamin B<sub>12</sub> accumulated within the cells; the vitamin B<sub>12</sub> is then got into a medium of  
 30 smaller volume by evaporation or adsorption, and the concentrate obtained in this way is then processed by further purifying steps to recover crystalline vitamin B<sub>12</sub>, or else feed-supplement compositions are prepared from the said concentrates.

These processes show the serious drawback that the vitamin B<sub>12</sub> which was originally included in the bacterium cells amounting approximately to only one per cent of the liquid fermentation product, is then released into a liquid of very large volume and must be subsequently converted  
 40 again into a concentrate of small volume by complicated concentrating operations involving losses of the active material. These drawbacks are especially important in the case of feed-supplement preparations, because the price of these is much lower

than that of crystalline vitamin B<sub>12</sub> and this lower price cannot bear the costs of the said concentrating operations.

To overcome these drawbacks, there have been introduced processes, wherein the microorganisms obtained in the fermentation liquid were precipitated by the addition of various protein-coagulating or precipitate-forming agents, flocculating or adsorbent additives, or by an appropriate  
 55 adjustment of the pH-value or similar operations, and the so-called bio-mass obtained in this way was then separated by filtration, decantation or centrifugation, and the so obtained and separated mass of relatively small volume was then processed  
 60 further to obtain a vitamin B<sub>12</sub> concentrate in order to recover crystalline vitamin B<sub>12</sub> therefrom or possibly to prepare feed-supplements containing vitamin B<sub>12</sub>.

The aim of the said processes is to bring the vitamin B<sub>12</sub> and other valuable materials into a small volume by precipitation. This method of concentrating may be advantageous in the case of preparation of crystalline vitamin B<sub>12</sub>, because the  
 70 unavoidable losses of the concentrating operation are reduced when the active material is recovered from smaller volumes and also the labour requirements of the process are lower. In cases, however, when  
 75 also feed supplements containing vitamin B<sub>12</sub> are to be prepared, the said advantages are accompanied by drawbacks too, because the precipitating agent or adsorbent used for the precipitation cannot be removed  
 80 economically from the obtained feed supplement, and its remaining therein is undesirable from the point of view of animal feeding; the presence of toxic chemicals or precipitating agents harmful for the health  
 85 of the animals (e.g. heavy metal salts used for the precipitation of proteins) makes the product totally inapplicable for feeding purposes; but also the non-toxic, inert precipitating agents or adsorbents make  
 90 undesired and often harmful, indigestible ballasts in feed supplement compositions.

We have found that the concentration of

vitamin B<sub>12</sub> to small volumes can be achieved very advantageously by the use of biologically utilizable adsorbents, i.e. adsorbent materials consisting of organic material digestible and biologically utilizable by the animals to which the produced B<sub>12</sub>-containing feed supplement will be administered. By the adsorption of the bacterium cells containing the produced vitamin B<sub>12</sub> in the fermentation broth by the aid of the said biologically utilizable adsorbent material, the separation of the entire B<sub>12</sub>-activity of the fermentation broth and its concentration into a small volume is substantially promoted and a concentrate is obtained in the form of an aqueous slurry which can be used after drying as a feed supplement of high nutritive value, but can be used equally well also for the recovery of crystalline vitamin B<sub>12</sub>. Fermentation liquids obtained by fermentations performed with Methanobacteria or with Propionibacteria, may be used equally well as starting materials for the process of the present invention.

Accordingly, the invention provides a process for the preparation of a dry vitamin B<sub>12</sub> concentrate of high protein content, useful as feed supplement or as starting material for the recovery of crystalline vitamin B<sub>12</sub> in which a fermentation broth containing vitamin B<sub>12</sub> is mixed with a non-toxic digestible organic adsorbent material as hereinafter defined, and the suspended solids included the adsorbent and the adsorbed bacterium cells containing the vitamin B<sub>12</sub> are then separated from the liquid and dried.

The term "organic adsorbent material" is used herein to denote non-toxic organic materials of cellular or particulate structure and consisting mainly of proteins and/or carbohydrates, digestible by the animals consuming the feed supplement, and having also in themselves a nutritive value and/or physiologically favourable properties. Aqueous starch suspensions, yeast suspensions, concentrated mycelium-suspensions of other fungi and similar digestible protein-containing cell suspensions of natural origin and free of toxic materials may be used as such biologically utilizable adsorbents. According to the experimental experiences, the said biologically utilizable adsorbents may be added in quantities of 0.01 to 5.0% by weight, preferably of 0.05 to 1.0%, to the fermentation broth; the said percentual values are related always to the dry material content of the added adsorbent.

In the course of the separation process, the particles of the biologically utilizable adsorbent carry with themselves the bacterium cells containing the vitamin B<sub>12</sub> and in this way an easy and quick sedimentation

thereof is achieved; practically the entire B<sub>12</sub>-activity of the fermentation broth containing originally only 1% dry material is so concentrated into an aqueous slurry containing 15 to 20% dry material, which contains besides the vitamin B<sub>12</sub>, growth factors and the protein content of bacterium cells originating from the fermentation broth, also the valuable organic materials of the biologically utilizable adsorbent. This so-called bio-mass, separated by sedimentation, decantation or preferably by centrifugal separation, is then dried in an appropriate equipment, e.g. in a rotary drier to yield a dry, well storable product of high vitamin B<sub>12</sub> and protein content, which can be used directly as feed supplement.

The concentrate obtained as a dry powder as described above may be used, however, also for the recovery of pure crystalline vitamin B<sub>12</sub> apt for human therapeutical use. For this purpose, an organic solvent able to coagulate the proteins and dissolve the vitamins, such as methanol, preferably 70% methanol is added to the dry powder, the pH-value of the suspension is adjusted to 8—9 by the addition of alkali, e.g. of a sodium hydroxide solution of 50 to 96%, preferably of 70% concentration, and the mixture is stirred for 30 to 40 minutes at elevated temperature, preferably 25° to 50°C; in this way the vitamin B<sub>12</sub> gets dissolved in the methanol. This extraction of the vitamin B<sub>12</sub> is performed preferably in the presence of 0.01 to 0.5% by weight ammonium rhodanide, benzaldehyde-cyanhydrin or ammonium formate. The obtained solution is then separated by decantation or filtration and evaporated; the crude vitamin B<sub>12</sub> obtained as evaporation residue is then purified in the usual manner, e.g. by the aid of an alumina column, and crystallized to obtain crystalline vitamin B<sub>12</sub> of pharmaceutical purity.

The invention is thus a process for the preparation of a dry vitamin B<sub>12</sub> concentrate of high protein content, useful as feed supplement and also as starting material for the recovery of crystalline vitamin B<sub>12</sub>, which is characterized in that a fermentation broth containing vitamin B<sub>12</sub>, obtained by fermentation with methanobacteria or propionibacteria, is mixed with a non-toxic digestible organic adsorbent material, preferably with an aqueous suspension of yeast cells, mycelia or starch granules in quantities of 0.01 to 5.0%, by weight preferably 0.05 to 1.0%, and then the suspended solids are separated by sedimentation and decantation or preferably by centrifugal separation and then dried. The product obtained in the form of a dry powder may be used as a feed supplement of high vitamin B<sub>12</sub> and protein

content in itself or after mixing it with other nutrient materials or feedstuffs known *per se*, but it may be used as a concentrate containing vitamin B<sub>12</sub> in high amounts and in an easily recoverable form, also for the recovery of crystalline vitamin B<sub>12</sub>.

The process of the present invention shows the following advantages over the processes presently used or known for the recovery of vitamin B<sub>12</sub> and/or for the preparation of feed supplements containing vitamin B<sub>12</sub>:

1. The process is substantially simpler than the presently used process which involves a series of operations of high labour requirements and material losses, e.g. boiling, filtering and precipitating.

2. The spacious and expensive plant equipment used in the present technological process, e.g. heat treatment reactors, filter presses are not needed to the new process; such equipments, if existing already in the plant, can be made available for other purposes.

3. The new process creates the possibility of producing more economically than hitherto a feed supplement of high nutritive value, high storage stability, favourable taste, odour and appearance, containing high amounts of digestible proteins as well as of vitamin B<sub>12</sub> and other growth factors.

4. The same product, which is obtained as a substantially ready-for-use feed supplement, can be used without any alteration as starting material for the preparation of crystalline vitamin B<sub>12</sub>; the process may be thus directed, as desired, to each of the two production lines with minimal requirements of equipment, labour and materials.

5. The process yields a product which can be worked up further in a technologically advantageous way as starting material for the production of vitamin B<sub>12</sub>.

6. The process may be used equally well in connection with the B<sub>12</sub>-producing fermentation process using Methanobacteria or Propionibacteria, for the processing of fermentation products obtained by using any suitable starting materials or nutrient-media.

The performance of the process of the invention is illustrated in more particulars by the following examples.

#### Example 1:

60 g of yeast (*Saccharomyces cerevisiae*) of 30% dry substance content, suspended in 400 ml of water, and 20 g of potato starch suspended in 100 ml of water were added to 10 liters of a fermentation broth obtained by fermentation with Methanobacteria containing on the whole 82 000 mcg of vitamin B<sub>12</sub> and microbiologically active factor III (as measured by paper chromatography), and the mixture was stirred vigorously. It

was then centrifuged for 1—2 minutes at 5 000 r.p.m. 452 g of a precipitate were obtained. This precipitate was then spread on plates, dried at 120°C, smashed up and ground and finally sifted. The weight of the obtained end product was 92.0 g.

1. g of this dry powder was suspended in 100 ml of water, the suspension was adjusted to a pH-value of 5.2 and boiled for 10 minutes. It was then centrifuged, the precipitate was discharged and in the liquid the content of microbiologically active cobalamins was measured by paper chromatography. The found value was 680 mcg/g. The 92.0 g of end product contained thus 62 320 mcg of active material. The yield was 86%.

#### Example 2:

The same amounts of yeast and starch as in Example 1 were added to 10 liters of a fermentation broth obtained by fermentation with Propionibacteria, containing 100 000 mcg/liter vitamin B<sub>12</sub>, and the precipitate was separated in the same way; 405 g of moist precipitate were obtained. By processing further and assaying as described in Example 1, a vitamin B<sub>12</sub> value of 890 mcg/g. was found in the obtained 81.0 g. of dry product of light gray colour; the total vitamin B<sub>12</sub> content of the product weighing 81.0 g. was thus 72 000 mcg. The yield was 72%.

#### Example 3:

By processing 10 liters of Methanobacteria fermentation broth as described in Example 1, 87.5 g. of dry end product containing a total of 50 700 mcg vitamin B<sub>12</sub> were obtained. This powder was mixed thoroughly with 450 ml. of 70% methanol in a one-liter round bottom flask, the pH-value of the mixture was adjusted to 8.5—9.0 by the aid of 30% sodium hydroxide solution, 0.1 g. of ammonium rhodanide was added to the mixture and the obtained mixture was kept for 35—40 minutes at 40 to 50°C on a steam bath under a reflux cooler, while stirring it continuously. After the extraction has been completed, the mixture was settled for 10 to 15 minutes and the obtained clear solution of red colour was decanted into a 500 ml suction filter flask; the precipitate was poured onto the suction filter and the solution retained in the precipitate was suctioned under water-jet vacuum to the bulk of the solution. The precipitate on the reaction filter was then washed with small portions of a total of 50 to 70 ml. of 70% methanol.

530 ml. of filtrate were obtained in this way. This solution was evaporated *in vacuo* to remove all methanol. The residual aqueous solution had a volume of 150 ml; its vitamin B<sub>12</sub> content, as measured by paper

chromatography, was 35 490 mcg/150 ml. The yield was 70%.

WHAT WE CLAIM IS:—

1. A process for the preparation of a dry vitamin B<sub>12</sub> concentrate of high protein content, useful as feed supplement or as starting material for the recovery of crystalline vitamin B<sub>12</sub> in which a fermentation broth containing vitamin B<sub>12</sub> is mixed with a non-toxic digestable organic adsorbent material as hereinbefore defined, and the suspended solids including the adsorbent and the adsorbed bacterium cells containing the vitamin B<sub>12</sub> are then separated from the liquid and dried.

2. A process as claimed in claim 1 in which the suspension consists of yeast cells, fungus mycelia and/or starch granules.

3. A process as claimed in claim 1 or 2 in which the fermentation broth is obtained by fermentation with *Methanobacteria* or *Propionibacteria*.

4. A process as claimed in any of the preceding claims in which the adsorbent material is added in a concentration of 0.01% to 5.0% by weight, calculated as the dry substance of the adsorbent.

5. A process as claimed in any of claims 1—4 in which the adsorbent material is added in a concentration of 0.05% to 1.0% by weight, calculated as the dry substance of the adsorbent.

6. A process as claimed in any of claims 1 to 4 in which the constituents of the adsorbent material are starch and yeast and it is added in a total concentration of 0.01% to 5.0% by weight calculated as the dry substance of the adsorbent.

7. A process as claimed in any of the preceding claims in which for the recovery of crystalline vitamin B<sub>12</sub>, the concentrate obtained in the form of dry powder is extracted at elevated temperature with an

organic solvent able to coagulate the proteins and dissolve the vitamin B<sub>12</sub>, and the vitamin B<sub>12</sub> is then recovered from the separated extract solution.

8. A process as claimed in claim 7 in which the organic solvent is methanol.

9. A process as claimed in claim 7 in which the organic solvent is 70% methanol.

10. A process as claimed in claim 7, 8 or 9 in which the extraction of vitamin B<sub>12</sub> is performed at a pH value between 8 and 9.

11. A process as claimed in any of claims 7 to 10 in which the extraction of vitamin B<sub>12</sub> is performed at a temperature of from 25°C to 50°C.

12. A process as claimed in any of the preceding claims in which the extraction is performed in the presence of 0.01 to 0.5% by weight of ammonium rhodanide, benzaldehyde cyanhydrin or ammonium formate.

13. A process as claimed in claim 1 substantially as hereinabove described.

14. A process as claimed in claim 1 substantially as described herein with reference to the Examples.

15. A dry concentrate containing vitamin B<sub>12</sub> and protein, useful as feed supplement and as starting material for the recovery of crystalline vitamin B<sub>12</sub> which comprises at least one organic adsorbent material which is starch, yeast or a fungus mycelia and vitamin B<sub>12</sub>-containing bacterium cells adsorbed on the said organic adsorbent material.

16. A dry concentrate containing vitamin B<sub>12</sub> and protein when prepared by a process as claimed in any of claims 1 to 14.

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